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13. ABSTRACT (Maximum 200 Words)

Expression profiling is a powerful novel technique to examine changes in the expression of a large number of genes at the same time. Different phenotypic states of a cell can be translated into specific gene expression signatures. As a complement to yeast two-hybrid studies we proposed using gene expression profiling to determine changes in gene expression as a function of expression of the *neurofbromatosis-2 (NF2)* gene in schwannoma cells. The strength of our approach is that we will not use tissues from patients, but will concentrate on cell lines in which NF2 expression can be controlled through the Tet/On system. In this system, treatment of cells with tetracycline (tet) induces expression of a tet-regulated gene, which in turn regulates the expression of the gene of interest. We have now generated several cell lines that express NF2 in a regulated fashion. The parent lines are RT4 schwannoma cells and mouse embryonic fibroblasts. A time course for NF2 expression has been established. A total of four cell lines have been tested on microarrays to detect expression changes. Surprisingly, no changes common to expression of isoform 1 and 2 have been detected so far. A manuscript describing our first set of experiments is in press.

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Introduction:

The NF2 protein, also called schwannomin or merlin, is a member of the ezrin-radixin-moesin family of proteins. Based on mutation analysis and protein expression studies, it is thought that NF2 is a tumor suppressor gene and that biallelic inactivation is required for phenotypic expression. Studies in cells that are NF2 deficient or that overexpress NF2 cDNAs have shown a powerful role of NF2 in regulation of cell morphology and proliferation. Despite these advances little is known about the action of NF2 in specific signaling pathways. This knowledge is of importance because it may lead the way to identifying treatments that modify these pathways.

Expression profiling is a powerful novel technique to examine changes in the expression of a large number of genes at the same time. Parallel analysis of gene expression reflects the changing view of signaling pathways towards signaling networks that have multiple and complex feedback and feed forward loops. Different phenotypic states of a cell can be translated into specific gene expression signatures. Expression profiling is not restricted to known genes, but changes in the expression of genes without known function can also be detected.

We are proposing to use gene expression profiling to determine changes in gene expression following regulated expression of NF2 cDNAs in schwannoma and meningioma cells. We will test the following hypotheses: 1. Regulated expression of NF2 results in specific changes in the expression profile at specific time points. 2. A subset of regulated transcripts differs between cells expressing NF2 isoform 1 and NF2 isoform 2. 3. NF2 induction results in partially shared expression changes in different cell types. 4. Expression changes are context dependent; expression profiles in confluent cells are different from non-confluent cells.

Body

Statement of Work Year 1

In the first year we will begin with the analysis of timed NF2 expression. In the second half of the first year we will examine the effects of overexpressing NF2 isoform 1 or isoform 2 in rat RT4 schwannoma cells.

For those genes that show consistent changes in expression we will perform quantitative northern blot analysis to validate expression changes.

We will continue the generation of meningioma cells. Lines expressing wildtype and mutant forms of NF2 will be generated in the Tet/On system.

We have established parameters for the induction of NF2 expression in RT4 cells. We had problems generating meningioma cells that stably express NF2. We therefore used mouse embryonic fibroblasts (MEF). cDNAs encoding NF2 isoforms 1 and 2 were put under the control of the tet-responsive promoter and introduced into MEF/3T3 TET-OFF cells using retroviral transduction.

These cells show a robust induction of NF2 expression at the RNA and protein levels after removal of doxycycline.

We have purified RNAs from RT4 and MEF cells and used these for expression profiling with the Affymetrix MG_U74Av2 chips. At a significance level of 0.05, we found that approximately 50 genes were regulated upon NF2 expression. This was true for either isoform. Interestingly, no regulated genes were shared between isoform 1 and 2.

We have now completed this study with the final conclusion that the overall number of regulated genes was less than the number expected by chance. Thus, we concluded that in the given experimental paradigm expression of NF2 did not induce any measurable effects on gene expression. These results are presented in detail in the appended manuscript that is now in press in the Journal of Cellular Biochemistry.

Statement of work year 2

Year 2

We will evaluate gene expression profiles in meningioma cells. Unless software updates are implemented at that time, we expect that comparison of human and rat profiles will be time-consuming and may have to be done manually.
For those genes that show consistent expression changes in meningioma cells we will perform quantitative northern blot analysis to validate expression changes.

Based on the results shown in our manuscript, we did not think that it was warranted to apply the same experimental paradigm to the study of meningeal cells. We have therefore changed our approach of detecting transcriptional changes making use of our recent findings that expression of NF2 and its binding protein HRS influence STAT-signaling in response to EGF (epidermal growth factor). STATS (Signal transducers and activators of transcription) are proteins that are important in intracellular signaling. STATs 3 and 5 have recently been linked to EGF signaling in tumor cells.

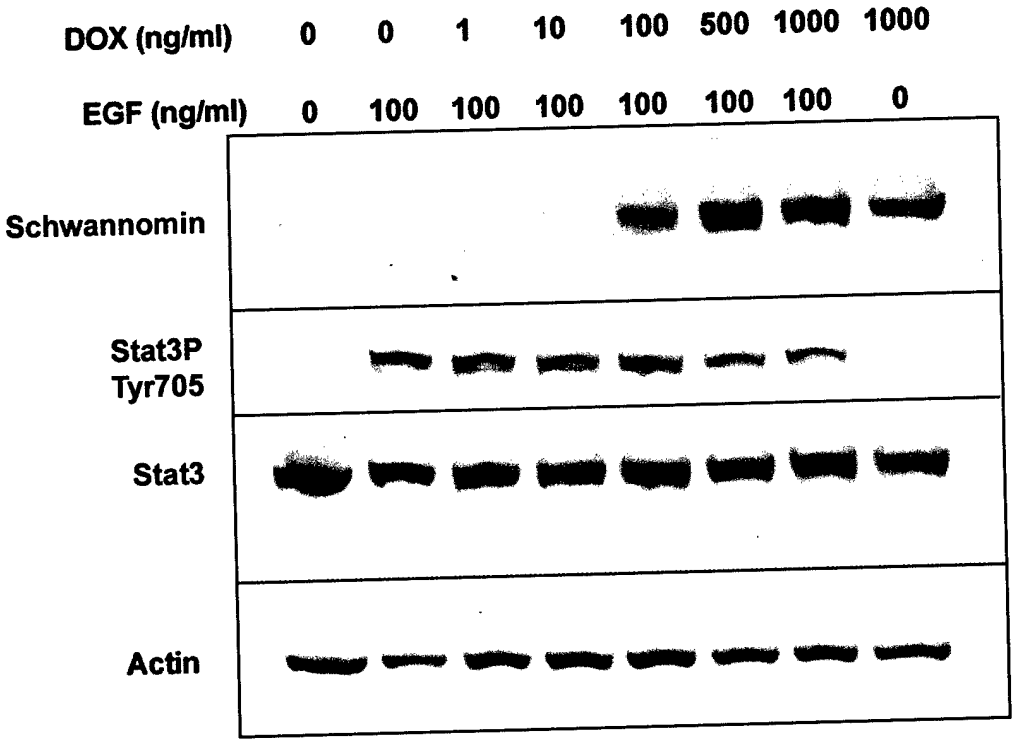

Figure 1: Effects of Schwannomin expression on EGF-mediated Stat3 activation in inducible Tet-on RT4/EGFR-GFP cells. Induced expression of schwannomin by treatment of schwannomin inducible Tet-on RT4/EGFR-GFP cells (line 10) with increasing doses of doxycycline inhibited EGF-mediated Stat3 phosphorylation. Schwannomin was detected using the anti-NF2 antibody A-19.

We have therefore applied for a no-cost extension of the funding period. We will establish a precise time-course for the action of EGF in our RT4 cells. Once optimal conditions have been established, we will perform expression profiling. We expect that treatment with EGF will induce strong changes in gene expression that will be modified by expression of NF2.

Key Research Accomplishments

- Establishment of cell lines that express NF2 under control of the TET-regulator.
- Establishment of array profiles for both NF2 isoforms.
- Establishment of a paradigm of STAT-activation in response to EGF.

Reportable Outcomes

Manuscripts in press: Oh MK & Pulst SM: Genetic heterogeneity of stably transfected cell lines revealed by expression profiling with oligonucleotide arrays. J Cell Biochem

Presentations

MK Oh et al. "The analysis of oligonucleotide microarray data"
DNA Microarray Workshop II, University of California, Los Angeles, CA. USA Sep. 16-17, 2002

MK Oh et al. "DNA microarray: A promising tool for pathway engineering"
Department of Chemical and Biological Engineering, Korea University. South Korea, May. 29, 2003

List of Personnel receiving pay from Research Effort

Stefan M. Pulst, MD
Min-Kyu Oh, Ph.D.
Pattie Figueroa, Research Associate

Conclusions

Gene expression profiling is still in its infancy. We are beginning to answer some basic questions regarding the use of stably transfected cell lines and the role of schwannomin and HRS. We have now established that quite to our surprise changes in steady-state levels of NF2 or HRS show relatively few effects on the expression of many genes.

We have now altered our strategy and are currently examining whether we can detect changes in the expression profile acutely after treatment with growth factors. These results will provide a framework to evaluate the alterations seen in human tumors associated with NF2 mutation.

APPENDIX COVER SHEET

1 Manuscript

JCB-03F-048

Journal of Cellular Biochemistry 9999:1–11 (2003)

Genetic Heterogeneity of Stably Transfected Cell Lines Revealed by Expression Profiling With Oligonucleotide Microarrays

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Abstract Large-scale gene expression measurements with oligonucleotide microarrays have contributed tremendously to biological research. However, to distinguish between relevant expression changes and falsely identified positives, the source and magnitude of errors must be understood. Here, we report a source of biological variability in microarray experiments with stably transfected cell lines. Mouse embryonic fibroblast (MEF/3T3) and rat schwannoma (RT4) cell lines were generated to provide regulatable schwannomin expression. The expression levels of 29 samples from five different mouse embryonic fibroblast clonal cell lines and 18 samples from 3 RT4 cell lines were monitored with oligonucleotide microarrays. Using hierarchical clustering, we determined that the changes in gene expression induced by schwannomin overexpression were subtle when compared with those detected as a consequence of clonal selection during generation of the cell lines. The hierarchical clustering implies that significant alterations of gene expression were introduced during the transfection and selection processes. A total of 28 genes were identified by Kruskal–Wallis rank test that showed significant variation between clonal lines. Most of them were related to cytoskeletal function and signaling pathways. Based on these analyses, we recommend that replications of experiments with several selected cell lines are necessary to assess biological effects of induced gene expression. J. Cell. Biochem. 9999: 1–11, 2003.

Key words: oligonucleotide microarray; expression profiling; biological variability; neurofibromatosis 2; schwannomin; merlin

Expression profiling with oligonucleotide microarrays has greatly influenced biological research. By measuring expression levels of thousands of genes simultaneously, microarray experiments facilitate the study of complicated phenotypes, such as cancer classification or circadian biological clocks [Golub et al., 1999; Storch et al., 2002]. This technology has also been employed to identify genes in cell culture model systems that reflect the function of transcriptional regulators, such as p53 [Zhao et al., 2000], EGR1 [Svaren et al., 2000], E2F [Ma et al., 2002], and Pax3 [Mayanil et al., 2001], or to characterize the effects of pathogenic protein expression, such as BRCA1 [Welcsh et al., 2002] and huntingtin [Sipione et al., 2002].

Because the number of genes simultaneously studied is large in microarray experiments, it is important to characterize the sources and

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magnitudes of error in an effort to sort false positives and negatives from truly regulated genes. Systematic analyses of errors inherent in oligonucleotide microarray experiments have just begun. Generally, 'technical (or experimental) variability' is defined as the errors incorporated during experimental steps, such as chip-to-chip variations, mRNA purification, cDNA probes synthesis, hybridization, scanning, and image analysis. Although this technical variability is reduced by the standardization of experimental protocols, a substantial level of noise can still occur during experimental procedures. A recent study with oligonucleotide microarray experiments showed that the hybridization step is the major source of the technical variability. The hybridization noise is highly dependent on the expression level of the genes, suggesting that the significance of gene expression fold changes must be adjusted depending on the gene expression levels [Tu et al., 2002].

In contrast, 'biological variability' originates from different sources of RNA, such as different tissue samples in a replicated experiment. In general, the biological variability is significantly higher than the technical variability [Bakay et al., 2002; Novak et al., 2002]. For example, significant background variations in expression levels were observed among mice that were genetically identical and had been housed under the same conditions. Many of these differentially expressed genes in those mice were heat shock, immune responsive, and hormone-regulated genes [Pritchard et al., 2001; Novak et al., 2002]. In other experiments, the expression levels varied significantly when the samples were taken from different regions of the same muscle tissue [Bakay et al., 2002]. These results suggest that the biological samples even in replicated experiments may contain significant background variation in gene expression levels.

Here, we report significant biological variability detected in stably transfected clonal cell-lines. With oligonucleotide microarrays, we monitored the expression level changes of several different clones selected for the regulated expression of the neurofibromatosis 2 (NF2) tumor suppressor protein, schwannomin (or merlin). Hierarchical clustering of the expression data showed that the expression profiles from different clonal lines were significantly different and these differences were not significantly influenced by expression of the transgene. Our results suggest that significant gene expression heterogeneity is introduced during the process of selecting stably transfected clones. This variation may be greater than that resulting from expression of the transgene of interest.

MATERIALS AND METHODS

Cells and Transfection

Full-length cDNAs coding for schwannomin isoforms 1 and 2 [Scoles et al., 1998] were cloned into the pRevTRE plasmid (Clontech) under the Tet-inducible promoter. The plasmids were transfected into a packaging cell line PT67 (Clontech) using Superfect reagent (Qiagen). The retrovirus produced from the packaging cell lines was infected into mouse embryonic fibroblast Tet-Off Cell line (Clontech). Hygromycin resistant Tet-Off MEF/3T3 clones were selected, diluted, and plated in 96-well plates to isolate single cell clones. The cells were incubated until confluent and seeded into three 96-well plates, two plates for testing schwannomin inducibility by dox/no dox comparison and a third for propagation. From 200 colonies, four cell lines (15-7 and 4-6 for isoform 1, and 23-6 and 2-19 for isoform 2) showing the best induction and suppression of schwannomin expression in dox/no dox comparisons were chosen for further experiments. The four selected cell lines and the parental MEF/3T3 Tet-Off cell line were maintained in DMEM containing 10% Tet-approved FBS (Clontech), 2 μg/ml doxycycline, 100 μg/ml G418, and 250 μg/ml Hygromycin B.

The construction and maintenance of inducible Tet-On RT4 cell lines for schwannomin and missense mutant (L64P) schwannomin were described previously [Gutmann et al., 2001].

RNA Preparation and cDNA Probe Synthesis

MEF/3T3 cell lines were incubated at 50% confluence in 100-mm plate and washed twice with 37°C dox-free media. Cells were reincubated for 24 h in medium with or without doxycycline for repression and induction of schwannomin expression. Total RNAs were purified from the cells using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. For immunoblotting experiments, 10% of cell lysates were saved. Labeled cRNA probes were generated from 10 μg of total RNA and subjected to microarray experiments using the MG_U74Av2 GeneChip (Affymetrix) as

described previously [Luthi-Carter et al., 2002]. For minimizing variability during the experiments, all cell lines were grown under the same conditions at the same time. The RNA purification and chip experiment were performed at the same time by the same person.

RT4 cell lines were incubated in media containing tetracycline-free fetal bovine serum (Clontech) until they reached 50% confluence and 1 μg/ml doxycycline was added into the media to induce schwannomin expression. Total RNAs were purified at 0, 6, and 24 h after the addition of doxycycline and subjected to the microarray experiments using RG_U34A GeneChip (Affymetrix).

Data Analysis

Array images (CEL files from the GENECHIP program, Affymetrix) were imported into the DCHIP program and the expression levels were calculated using the model-based method after normalization with DCHIP's rank invariant protocol [Li and Wong, 2001]. Gene filtering based on the intensities or 'present' call and Student's *t*-test were performed using the DCHIP program. The *t*-test was also performed with the expression levels calculated by the Affymetrix GENECHIP program. The results were very similar to those obtained using the DCHIP protocol (data not shown).

For cluster analysis and Kruskal–Wallis rank test, the expression levels from DCHIP were divided by the average expression level of the respective gene in the entire set of chips (26 chips for MEF/3T3 cell line and 17 chips for RT4 cell line). The expression ratios were imported into the CLUSTER program [Eisen et al., 1998], log-transformed and analyzed by the average linkage clustering method. The clustered tree was drawn by the TREEVIEW program [Eisen et al., 1998]. The log-transformed expression ratios were used for Kruskal–Wallis rank test, a non-parametric analysis method similar to one-way analysis of variance (ANOVA) to reveal the differentially expressed genes in different cell line clones. Kruskal–Wallis rank test was conducted using the S-Plus2000 program (Insightful Corp.).

Immunoblotting

The cell lysates from MEF/3T3 cell line samples were precipitated by adding five volumes of acetone (−20°C), incubating for 15 min at −20°C, and centrifuging for 20 min at 4°C. The pellets were air-dried and dissolved in 20 μl sample buffer consisting of 50 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 50 mM 2-mercaptoethanol, and 0.1% bromophenol blue. The protein samples were electrophoresed on 4–15% SDS–PAGE gradient gels (BioRad) and transferred to nitrocellulose membrane. The immunoblots were detected with anti-schwannomin antibody 2781 described previously [Huynh and Pulst, 1996]. The blots were reprobed with anti-actin monoclonal antibody AC40 (Sigma) for normalization of protein loading. In RT4 cells, schwannomin or mutant schwannomin was detected in a similar manner except that the anti-schwannomin antibody WA30 was used [Gutmann et al., 1997].

RESULTS

Generation of Inducible Schwannomin-Expressing MEF Cell Lines

Inducible cell lines expressing schwannomin protein isoforms 1 and 2 were generated in mouse embryonic fibroblast cells (parental MEF/3T3 Tet-Off lines obtained from Clontech) using retroviral infection. The four selected cell lines (4-6, 15-7 for isoform 1 and 2-19, 23-6 for isoform 2) and the parental cell line were maintained in 100-mm plates with 2 μg/ml doxycycline in the medium. When cells reached 50% confluency, cells were washed thoroughly in 37°C dox-free media to remove any trace of doxycycline and incubated again in the media with or without doxycycline for 24 h to repress or induce the schwannomin protein expression. The experiment was replicated for parental, 4-6, 15-7, and 2-19 cell lines and repeated four times for line 23-6 (Table I). The repeated experiments were denoted with small characters, a, b, c, and d after cell line names. Based on immunoblotting analysis (Fig. 1), several samples, such as 15-7b, 4-6a, 4-6b, 23-6a, and 23-6c, showed low basal levels of schwannomin expression and strong induction of schwannomin upon doxycycline withdrawal.

The purified RNAs were labeled and hybridized for expression profiling using Affymetrix MG_U74Av2 chips. After hybridization and image analysis, the image files (CEL files) were imported into the DCHIP program [Li and Wong, 2001]. While calculating expression levels using the PM/MM difference model, the program excluded the probe sets that did not match the overall pattern of intensities

TABLE I. Microarray Experiments Using 29 Mouse Embryonic Fibroblast Tissue Samples From Five Cell Line Clones

Clonal cell line	Array name	P call (%)	Array outlier (%)	Single outlier (%)	Flag
Parental cell	P	51.5	0.288	0.263	
	PaD	50.6	0.416	0.260	
	PaN	50.3	0.625	0.271	
	PbD	52.1	0.296	0.231	
	PbN	48.7	3.820	0.903	*
MEF NF2 isoform 1 clone 4-6	4-6	52.3	0.480	0.266	
	4-6aD	49.4	0.400	0.284	
	4-6aN	51.5	0.288	0.200	
	4-6bD	46.0	3.692	1.025	*
	4-6bN	53.0	0.312	0.234	
MEF NF2 isoform 1 clone 15-7	15-7	49.2	0.681	0.417	
	15-7aD	50.2	0.464	0.336	
	15-7aN	49.1	0.496	0.332	
	15-7bD	51.8	0.673	0.244	
	15-7bN	52.2	0.288	0.212	
MEF NF2 isoform 2 clone 2-19	2-19	51.7	0.488	0.197	
	2-19aD	50.5	0.248	0.200	
	2-19aN	51.0	0.280	0.294	
	2-19bD	50.2	0.553	0.240	
	2-19bN	53.4	0.320	0.226	
MEF NF2 isoform 2 clone 23-6	23-6	51.2	0.208	0.173	
	23-6aD	52.0	0.488	0.339	
	23-6aN	47.7	1.241	0.542	
	23-6bD	49.1	0.416	0.307	
	23-6bN	46.3	3.211	0.952	*
	23-6cD	52.8	0.537	0.388	
	23-6cN	52.0	0.569	0.380	
	23-6dD	52.5	0.641	0.334	
	23-6dN	52.1	0.288	0.266	

The cell lines used were parental (P), 4-6, 15-7, 2-19, and 23-6. Array names ending with N are the samples from 24 h after the removal of doxycycline, and ones with D are the control sample with doxycycline in the medium. The small characters (a–d) before N or D of the array name represent the repeats of experiments. Samples without N or D were taken at 0 h before removal of doxycycline at 50% confluence. P call (%) represents percentage of the probes called 'present' in the array by DCHIP program. Array outliers represent the probe sets that do not follow the overall pattern of the intensities calculated by model-based expression tags in DCHIP. Single outliers represent single probes that do not follow intensity pattern of the respective probe in other sets. If the percentages of array and single outliers are high, the array is flagged.

observed with other chips or the probes did not match the intensity pattern of the respective probe on other chips. Those probe sets were designated "array outliers" or "single outliers," respectively (Table I). In general, the outlier percentage is inversely correlated with the image quality [Schadt et al., 2000]. Among the 29 microarray experiments, three chips, P2N, 4-62D, and 23-62N, showed notably higher percentages of probe/single outliers than others. Therefore, these three chips were eliminated from subsequent analyses.

Effect of Schwannomin Induction on Expression Profiling

Schwannomin expression was monitored with immunoblotting experiments (Fig. 1). Two samples each for isoform 1 (4-6a, 15-7b) and isoform 2 (23-6a, and 23-6c) showed strong induction and repression of schwannomin expression responsive to the level of doxycycline. Two pairs of expression profiles, 4-6aD/4-6aN and 15-7bD/15-7bN, were analyzed using Student's *t*-test in the DCHIP program to identify the genes regulated by schwannomin isoform 1 expression. For pair-wise comparison, we first identified the genes called 'present' in at least one chip by the DCHIP program. In all pair-wise comparison, approximately 7,000 genes were used for further analysis. At significance levels of $P < 0.05$ and < 0.01, one would expect to identify 350 and 70 false positives, respectively, from a set of 7,000 probes. We found only one gene to be significantly regulated in the 15-7bD/15-7bN comparisons and none in the 4-6aD/4-6aN comparisons using a significance level $P < 0.01$. With a relaxed stringency ($P < 0.05$), 48 and 53 genes were filtered out as changed in the 4-6aD/4-6aN and the 15-7bD/15-7bN comparisons, respectively. In summary, the number of genes significantly changed was much smaller than the expected number of false positives, suggesting that expression levels of very few or no genes were significantly changed.

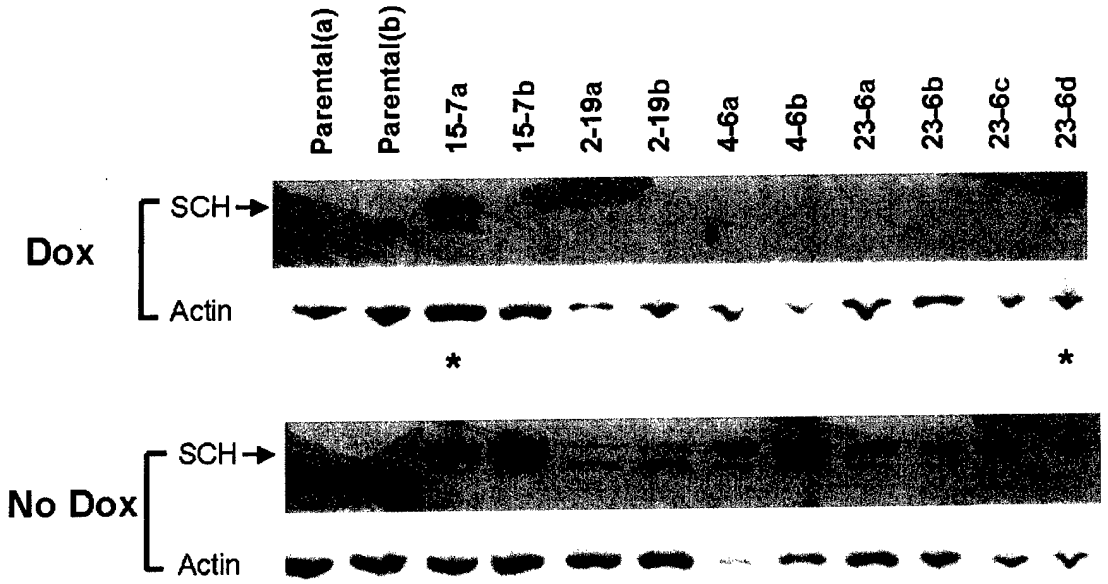

Fig. 1. Western blot detection of schwannomin expression levels in MEF/3T3 cell lines. Cells were incubated in the medium with or without doxycycline for 24 h followed by washing with dox-free medium. After electrophoresis of total cell lysate, schwannomin was detected by using antibody 2781 [Huynh and Pulst, 1996]. To control for loading, blots were reprobed with an anti-actin monoclonal antibody AC40 (Sigma). Detail experimental procedure is described in the text. Parental cell line samples show endogenous expression level of schwannomin. Two cell line samples, 15-7a and 23-6d, show poor suppression under the existence of doxycycline and marked with asterisk.

To detect genes differentially expressed in clonal cell lines, we also examined the number of regulated genes between the two control (uninduced) samples (4-6aD/15-7bD) and between the two induced samples (4-6aN/15-7bN). Surprisingly, these analyses found 201 and 159 differentially regulated genes using *t*-test with $P < 0.05$, respectively. Among them, 62 genes were commonly up- or downregulated in both comparisons. This suggests that many genes were differentially expressed between 4-6a and 15-7b cell lines even before the schwannomin isoform 1 was induced. The differential expression profiles between different clonal cell lines will be discussed below.

Expression of schwannomin isoform 2 did not result in significant gene expression changes. According to Student's *t*-test in the DCHIP program, none of the probes was significantly regulated with a value of $P < 0.01$ in comparisons between the 23-6aN/23-6aD and between the 23-6cN/23-6cD chip sets. With a *P*-value < 0.05, 83 and 18 genes, respectively, were differentially changed, numbers smaller than the expected false positives. Among them, five genes were regulated in common, but their expression level changes were not significant (<1.5-fold). A similar number of differentially regulated genes were found when we compared two replicate samples from the same cell line before and after induction. This suggests that neither schwannomin isoform caused significant expression changes in a large number of genes when overexpressed in mouse embryonic fibroblasts.

Experimental Design and Expression Changes in RT4 Cells

The effect of regulated schwannomin expression was independently examined in rat RT4 schwannoma cells. For these experiments, two cell lines, in addition to the parental Tet-On RT4 line, were used; one line expressed wild type schwannomin, the second one schwannomin with the disease-associated L64P amino acid substitution [Gutmann et al., 2001]. Previous studies have demonstrated that schwannomin containing the L64P mutation is functionally inactive [Gutmann et al., 2001]. Upon addition of doxycycline to the media, schwannomin was consistently induced (Fig. 2). Total RNAs were purified from the cells at 0, 6, and 24 h after induction; the experiment was replicated and the samples designated by the small letters a

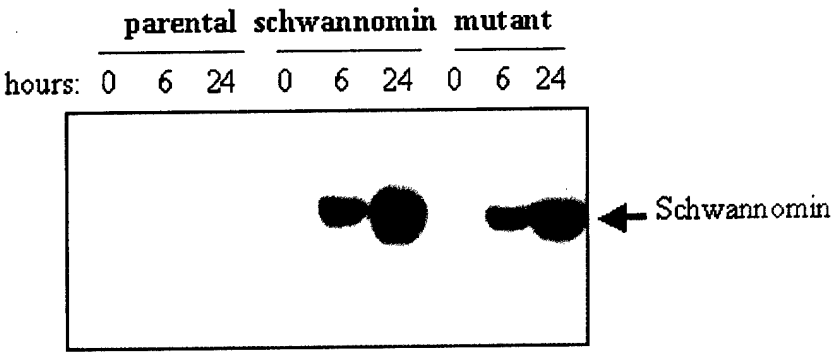

Fig. 2. Western blot analysis of protein expression in RT4 cell line clones. Parental cell line showed endogenous schwannomin expression, while cell lines transfected with schwannomin and mutant schwannomin show significantly induced protein expression after adding doxycycline. The proteins were probed with WA30 antibody (4).

or b. The expression levels of the 18 samples were monitored using the Affymetrix RG_U34A chips (Table II). In this experiment, M0b showed a high array/single outlier percentage and was excluded from subsequent analyses.

The effect of schwannomin or mutant schwannomin induction on gene expression profiles was analyzed in the same way as the MEF/3T3 cell lines. The expression levels of 0-h samples from parental, schwannomin, and mutant schwannomin producing cell lines were compared with 6- or 24-h samples of the same cell lines using the *t*-test. About 4,500 probe sets were called 'present' in more than one chip in each pair-wise comparison. By the *t*-test with $P < 0.05$, 275 false positives would be expected. The differentially expressed genes found by the *t*-test between 0-h samples and 6- or 24-h samples did not exceed the expected number of false positives in any comparison. For example, the comparison between 0- and 24-h samples of schwannomin inducible cell line (S0a/S24a in Table II) found 34 differentially expressed genes. This number is even smaller than 67 and 37 genes found by the replicate sample comparisons (between S0a/S0b and between

TABLE II. Microarray Experiments Using 18 RT4 Cell Line Samples

Clonal cell line	Array name	P call (%)	Array outlier (%)	Single outlier (%)	Flag
Parental RT4 cell	P0a	46.9	0.511	0.100	
	P0b	47.3	0.557	0.079	
	P6a	49.9	0.636	0.073	
	P6b	49.7	0.443	0.064	
	P24a	48.3	0.273	0.045	
	P24b	49.7	0.284	0.071	
Schwannomin inducible RT4 cell	S0a	46.1	0.443	0.084	
	S0b	46.1	0.466	0.104	
	S6a	47.8	0.784	0.143	
	S6b	47.7	0.386	0.043	
	S24a	48.4	0.955	0.130	
	S24b	48.3	0.341	0.071	
Mutant schwannomin inducible RT4 cell	M0a	47.7	0.227	0.054	
	M0b	44.3	5.455	0.870	*
	M6a	47.6	0.841	0.136	
	M6b	47.6	1.978	0.319	
	M24a	47.0	0.330	0.073	
	M24b	46.7	0.580	0.126	

Array names starting with P, S, and M represent parental, schwannomin-, and mutant schwannomin-expressing cell lines, respectively. The numbers following the letter represent the sampling time after the induction of protein expression, and a and b the replication of the experiment. The remainder of the terms are the same as in Table I.

S24a/S24b), which indicates that schwannomin expression had little effect on gene expression profiles in RT4 cells.

Hierarchical Clustering of the Data

Instead of comparing differentially expressed genes by *t*-test, the overall pattern of the expression profiles was investigated with cluster analysis in MEF cells. Among 12,300 probe sets on the Affymetrix MG_U74Av2 chip, 7,185 probe sets were chosen after eliminating the probe sets called 'absent' in more than 80% chips by DCHIP among the 26 chip data. The expression levels of those probe sets were analyzed by an unsupervised cluster analysis. The expression profiles formed five major clusters. Each cluster contained the samples originating from the same clonal cell lines (Fig. 3a). This indicated that samples expressing schwannomin did not cluster together, but instead that clonal selection during the generation of stably expressing cell lines had resulted in distinct expression profiles.

The cluster analysis was re-examined, this time focusing on probes with high expression levels that[Q3] are known to be more reliable [Tu et al., 2002]. We chose 958 probe sets with intensities over 500 (maximum intensity ~ 6400) and presence in more than 80% of chips. The hierarchical clustering revealed a similar cluster tree pattern to the one with 7,185 probe sets, each demonstrating five clusters with samples from different cell lines. Minor exceptions were identified: the sample 4-6aD clustered with the 15-7 cell line samples and the sample P clustered with the sample 2–19 (Fig. 3b).

Cluster analysis with the expression profiles of RT4 cell lines showed similar results. Among the 8,700 probes on the RG_34A chip, 4,689 probe sets were called 'present' in more than 20% of the chips. The cluster analysis with the expression profiles of the probe sets showed that 17 samples formed three major clusters with each cluster containing the samples from the same clonal cell line (Fig. 4). The time points after the protein induction had few effects on global gene expression levels, although the 6- or 24-h samples expressed significant levels of wild type or mutant schwannomin expression (Fig. 2). This ascertained that the variations incorporated during selection process were more significant than the ones induced by schwannomin protein overexpression.

Differentially Expressed Genes in Clonal Cell Lines

To identify an even smaller subset of differentially expressed genes in different clonal cell lines, Kruskal–Wallis rank test was performed with log-transformed gene expression ratios.

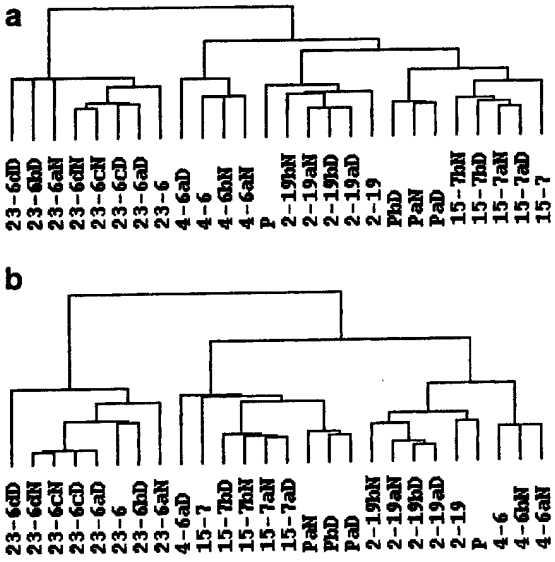

Fig. 3. Unsupervised hierarchical clustering of expression profiles of 26 mouse embryo fibroblast cell line samples listed in Table I. The cluster analysis was performed with (a) 7,185 probe sets and (b) 958 probe sets selected based on the percentages of present calls and/or expression levels as described in the text. The length of vertical bars indicates the degree of difference in gene expression levels between samples. The samples from the same cell line clone generally cluster together irrespective of the induction of schwannomin protein expression.

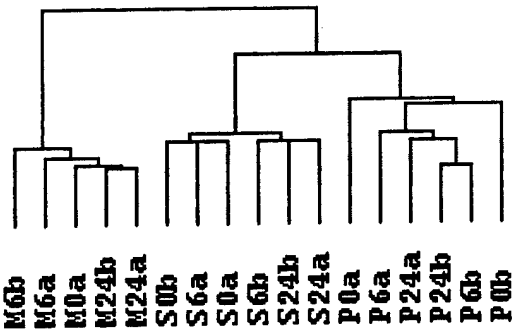

Fig. 4. Unsupervised hierarchical clustering of gene expression data of 17 RT4 cell lines in Table II. The clustered tree shows that different cell line clone is the major variable that makes differences in the expression levels. Among the cell line clones, parental and schwannomin transfected cell lines show similar expression levels and missense mutant schwannomin transfected cell line shows[Q4] the most distinct expression profile.

The expression levels of the samples from the same clonal cell line were grouped together, generating five and three groups for experiments with MEF/3T3 and RT4 cell lines, respectively. The genes differentially expressed in different groups were searched for with Kruskal–Wallis rank test. The analyses with MEF/3T3 and RT4 cell lines selected 320 and 292 genes, respectively, as "highly expressed" (intensities over 500 and 'present' in more than 80% of the chips) and as "significantly varied" ($P < 0.01$ in Kruskal–Wallis rank test) in different clonal cell lines. A total of 28 genes variable in both mouse and rat cell lines were identified in this fashion. Of these transcripts, 13 genes were identical for both cell types and 15 were highly related. Of the 28 genes, a significant number represented cytoskeletal related genes (8 out of 28) including actin, annexin A1 (lipocortin-1), fibronectin, integrin, osteonectin, and tubulin. The remaining genes included cytochrome-c oxidase subunits, proteasomal proteins, and ribosomal proteins. Most of the 28 genes varied less than 2-fold among the cell lines with the highest and lowest measurements. However, a few genes, such as proteasomal subunit α and protein phosphatase 4, were differentially expressed up to threefold among the RT4 cell line clones. The list of 28 genes and their expression levels are shown in Figure 5.

DISCUSSION

Subtle Expression Level Changes by Schwannomin Inductions

We performed microarray experiments with two stably transfected cell lines, MEF/3T3 and RT4, expressing schwannomin under the control of an inducible promoter. Our experiments could not identify significantly regulated genes induced by schwannomin overexpression. This was unexpected, considering that schwannomin is a tumor suppressor protein involved in signal transduction pathways mediated by Rho/

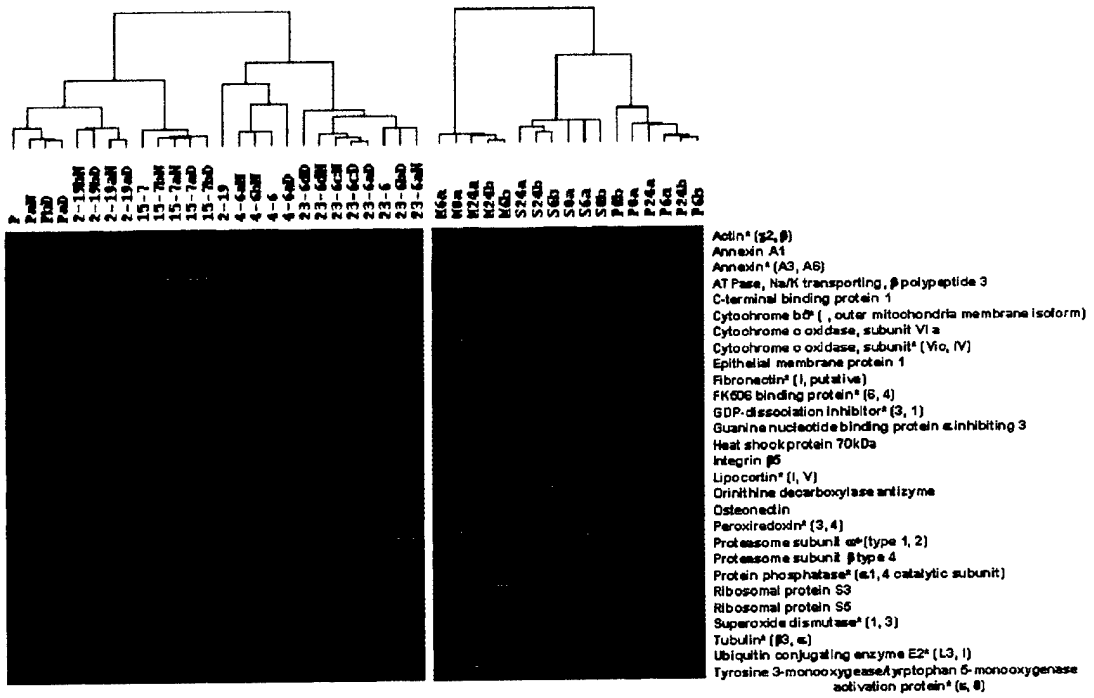

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Fig. 5. The list of the genes varied significantly in different MEF/3T3 and RT4 cell line clones. The genes were chosen using Kruskal–Wallis rank test as described in the text. Bright red and green colors represent the expression level more than 50% above and below the average level in all samples, respectively. The[Q5] darker the color, the lesser the change in expression level. The left clustering tree is the expression profile of MEF/3T3 cell line samples, while the right one is of RT4 cells. The samples from the same clonal cell lines were clustered together except the sample 2–19. Among the genes presented in this figure, 13 are the functionally identical genes in both cell lines and the other 15 with asterisk at the end of gene name are the genes with similar function according to the mouse and rat gene annotations. The first term in the parenthesis is the gene name corresponding to MEF/3T3 cell and the second to RT4 cell.

Rac and STAT3/5 [Pelton et al., 1998; Scoles et al., 2002]. It is possible that changes in expression levels were so subtle that they were not detected in chip hybridizations or that the relevant genes were not represented on the chips used in our experiments. In addition, gene expression changes may have been missed, because they were asynchronous or occurred outside the time frames that we examined. It is also possible that the major effects of schwannomin overexpression result in direct protein modification without significantly altering steady-state gene expression profiles.

Another possibility is that transcriptional changes induced by schwannomin are only noticeable under certain experimental conditions in vitro. For example, we previously showed that schwannomin regulated IGF-I induced STAT3 phosphorylation after serum-starvation [Scoles et al., 2002]. Phosphorylated-STAT3 binds to DNA and alters expression levels of many genes. The experimental condition used in this study may not have been appropriate to detect those physiological changes. Finally, whereas the loss of schwannomin protein causes significant physiological effects such as the formation of tumors in the peripheral nervous system, the induction of schwannomin protein may not cause any severe physiological effects in cells maintained in culture. Indeed, cDNA microarray data with human schwannomas showed dramatic transcriptional changes, such as upregulation of osteonectin and RhoB GTPase as well as downregulation of LUCA-5 and CDK2 [Lasak et al., 2002; Welling et al., 2002]. Expression level changes in human schwannomas in those studies require further verification. Small sample size, tissue heterogeneity, and inter-individual variations among human patients may result in a substantial rate of false positives [Bakay et al., 2002].

Microarray experiments have not always been able to identify gene expression changes despite the presence of significant phenotypes. Detailed microarray experiments with mouse brains lacking the MECP2 gene found no significant transcriptional changes [Tudor et al., 2002]. The result was even more remarkable, as MECP2 is known as a transcriptional repressor and its mutation causes a severe cognitive phenotype in human, Rett syndrome. Our microarray experiments strongly suggest that schwannomin overexpression in two different cell types caused few transcriptional changes as well.

Heterogeneity Introduced by Clonal Selection

Transfection of a gene of interest into cultured cells is one of the most commonly used methods to study the cellular function of the respective protein. The high-throughput screening ability of microarray experiments has provided a powerful tool to characterize protein function in transfected cell lines. Various experimental designs have been used, such as transient transfection or stable transfection with inducible or constitutive promoters. However, the background variability introduced by transfection or the selection process has not been systematically studied.

With microarray experiments of stably transfected cell lines, we observed significant alterations of gene expression between different clonal cell lines. The unsupervised cluster analyses with 26 MEF/3T3 and 17 RT4 cell line samples indicated that clonal selection was the major variable that changed gene expression levels independent of expression of exogenous schwannomin. Although technical variations could confound the results, they would not contribute much to the observed variations, because in each experiment, RNA purifications were carefully performed under the same conditions and all chip experiments were performed on the same day in random orders. In addition, replicate samples from the same cell line and treatment condition (indicated by small letters in Figs. 3 and 4) clustered together indicating a high degree of reproducibility of the data.

We used Kruskal–Wallis rank test to identify a small subset of genes that were highly variably expressed across all different clones including mouse and rat cell lines. This small set of 28 genes resulted in a tree that was highly similar to the ones generated with a larger number of genes. Inspection of the expression patterns of single genes indicated that expression was highly different between different cell line clones. For example, annexin A1 expression was high in 23-6 cells, but low in mouse parental cells and in 15-7 cells. The inverse was observed for peroxiredoxin that showed higher expression in all 15-7 clones compared with 23-6 clones independent of schwannomin expression.

The expression of many cytoskeletal proteins varied in expression between the different clones. It should be noted that some of these proteins, such as actin and tubulin, are being used as reference proteins to normalize the gene or protein expression levels in Northern or Western blots. Our results strongly suggest that the expression levels of these genes may vary in different clonally derived cell lines, although the level changes were less than 40% between the clones.

The changes in background expression may not be relevant when examining the effects of exogenously expressed genes that have a strong effect on gene expression. For example, a number of regulated genes as a result of overexpression of BRCA1 [Welcsh et al., 2002] or mutant huntingtin [Sipione et al., 2002] were identified despite background variability of stably transfected cells. On the other hand, clonal background changes may greatly influence results, when exogenously expressed transgene have weak effects, because expression levels for some genes may greatly differ between cell clones that appear phenotypically highly similar.

The reason why substantial expression level changes are introduced during transfection or selection process requires further study, but may relate to the chromosomal location of plasmid integration or the selection of particular cells in the transfected pool with pre-existing subtle differences in background gene expression. This background variability should be considered during experimental design and data interpretation. In the mean time, our results suggest that microarray experiments with stably transfected genes should be replicated with multiple cell line clones and that the direct comparison of expression data from different clones should be interpreted with caution. Alternatively, transient transfection of the gene can reduce the background variability if the transfection method is highly efficient or if it is combined with a selection method.

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